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The pain of antisense: in vivo application of antisense oligonucleotides for functional genomics in pain and analgesia

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Received 25 April 2003; accepted 8 May 2003

Abstract

As the genomic revolution continues to evolve, there is an increasing demand for efficient and reliable tools for functional characterization of individual gene products. Antisense oligonucleotide-mediated knockdown has been used successfully as a functional genomics tool in animal models of pain and analgesia yet skepticism regarding the validity and utility of antisense technology remains. Contributing to this uncertainty are the lack of systematic studies exploring antisense oligonucleotide use in vivo and the many technical and methodological challenges intrinsic to the method. This article reviews the contributions of antisense oligonucleotide-based studies to the field of pain and analgesia and the general principles of antisense technology. A special emphasis is placed on technical issues surrounding the successful application of antisense oligonucleotides in vivo, including sequence selection, antisense oligonucleotide chemistry, DNA controls, route of administration, uptake, dose-dependence, time-course and adequate evaluation of knockdown.

Keywords: Knockdown; Central nervous system; Rat; Mouse; Gene targeting; Intrathecal; Animal model; Nociception

Contents

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0169-409X/03/$ - see front matter
doi:10.1016/S0169-409X(03)00108-4

1. Introduction

Antisense oligonucleotide (ASO)-mediated knockdown of gene expression has contributed substantially toward our understanding of the physiology and pathobiology of pain and analgesia. ASO use has many advantages including the ability to specifically target a single member of a large and/or highly homologous family and the ability to control the timing of protein knockdown. ASO technology therefore enables us to elucidate the role of a target protein in either the induction or maintenance of pathological processes by beginning the ASO treatment before or after induction, respectively.

ASOs are particularly well suited for the validation of new drug targets because the functional effects of ASO-mediated knockdown of a prospective drug target can predict its therapeutic potential. Through genomics we have increased our understanding of many diseases at the molecular level, yet the functional and therapeutic implications of much of the information remain unexplored. The need for efficient and reliable tools for the validation of potential new drug targets has become a critical issue in the drug discovery process.

ASO-based approaches have been successfully applied to many classes of pain-relevant genes including G-protein coupled receptors (GPCRs), voltage- and ligand-gated ion channels, neuropeptides and second messengers. However, despite the many important contributions, skepticism about the validity and utility of ASOs as a functional genomics tool remains. The general impression that the antisense technology, although initially very promising, has not lived up to expectations is somewhat justified. In looking at the number of Medline citations (Fig. 1) since 1985 in response to a keyword search

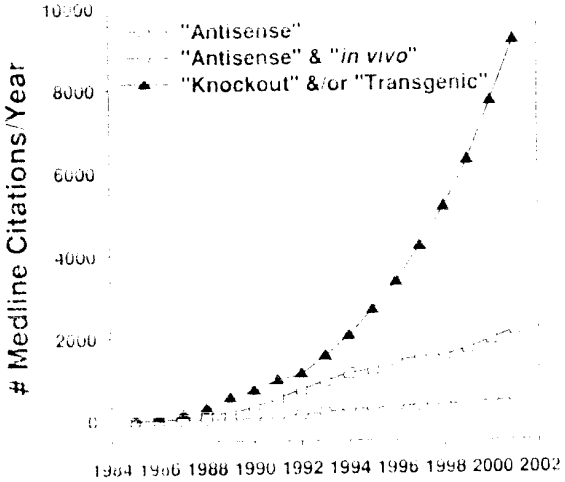

Fig. 1. Number of Medline citations containing the keywords "antisense", "antisense and in vivo", or "knockout or transgenic". The number of antisense related citations has remained relatively constant for more than a decade. In the same time period the number of publications containing the terms "knockout" and/or "transgenic" have risen rapidly.

for "knockout" or "transgene" we see a doubling every 2–3 years, suggesting continued increased utilization of the method. In contrast, examination of the number of Medline citations for "antisense" reveals minimal growth by comparison. Limiting the number of "antisense" citations to those with reference to "in vivo" produces a nearly flat line. This under-representation in the literature could be interpreted as indicative of fundamental flaws in the antisense technology. On the contrary, we suggest that the apparent under-utilization of ASOs is symptomatic of the many technical and methodological challenges of ASOs use and the relative lack of systematic studies delineating the best practices for in vivo applications. The goal of this review is therefore to outline the contribution of antisense studies to the field of pain and analgesia, the general principles of antisense technology, and the many technical issues surrounding the application of ASOs in vivo.

2. Antisense contributions to in vivo pain studies

The rationale driving the application of ASOs in pain studies generally falls into several major categories. First, ASOs enable the independent functional characterization of a single member of a closely related family of proteins that cannot be specifically targeted using pharmacological tools. Second, ASOs can be used to examine the molecular basis of pharmacologically defined subtypes (for example, see the discussion on opioid receptors in Section 2.2). Third, ASOs provide a tool for determining the mechanism of action of pharmacological agents. Fourth, as a functional genomics tool for target identification and validation, the antisense technology offers a screening method for potential new therapies and its use can streamline and accelerate the drug discovery process. Finally, ASOs have the potential to serve as therapeutic agents.

The studies highlighted in this section illustrate the wide variety of pain-relevant proteins that have been targeted with ASOs. Many of these studies have made significant contributions to our understanding of the neural mechanisms of pain and analgesia. This overview is not intended as a comprehensive tally of all the pain-relevant ASO based reports published to date, nor does it include studies of pain-relevant genes where the experimental endpoints did not include nociception. The specific details of the experimental design, the evaluation of knockdown and the pain models used in selected studies are itemized in Table 2 and Section 5.

2.1. Ion channels

2.1.1. P2X receptors

The P2X purinergic receptors (ATP-gated cation channels) can signal tissue damage when activated by ATP released from dying cells [1,2]. The P2X3 subunit in particular has been implicated in acute and chronic pain since its discovery [3–5], largely due to its localization in nociceptive sensory neurons [6–9]. However, the lack of specific pharmacological tools prevented the generation of direct evidence to support this contention. Studies using knockout mice reported a lack of involvement of P2X3 in normal somatic pain thresholds but hinted at some involvement in the signaling of more tonic stimuli [10,11]. It was not until the publication of two studies successfully targeting P2X3 with ASOs that the importance of this subunit in chronic neuropathic and inflammatory pain was demonstrated directly [12,13]. In inflammatory models, P2X3 appeared to be involved in Complete Freunds Adjuvant (CFA) but not carrageenan-induced thermal hyperalgesia, and the ability of P2X3 ASO-treatment to attenuate CFA-induced hyperalgesia correlated with the magnitude of the knockdown obtained in each animal [13]. Knockdown of P2X3 attenuated mechanical allodynia (assessed by von Frey filaments) induced by spinal nerve ligation [13] but not mechanical allodynia induced by partial sciatic nerve injury [12]. However, the development and maintenance of mechanical hyperalgesia following partial sciatic nerve injury, measured using an analgesymeter (Ugo-Basil Milan, Italy), was attenuated by P2X3 knockdown [12]. In contrast, P2X3 knockout mice show impaired thermal but not mechanical allodynia following nerve injury, suggesting that the P2X3 receptor is not involved in mechanical pain [14]. These inconsistencies could be due to strain, species or methodological differences. Despite these discrepancies, however, ASO approaches have clearly provided direct

evidence for a role of P2X3 receptors in chronic pain.

2.1.2. Voltage-gated sodium channels

Nerve injury results in an increased density of voltage-gated Na^+ channels in the damaged nerve, leading to abnormal excitability [15]. The relative importance of the members of the voltage-gated Na^+ channel family, NaV1, in chronic pain remains a subject for debate. Although there are no pharmacological tools available for the selective targeting of each subtype, based on their sensitivity to tetrodotoxin (TTX) the channels have been classified into two functional categories: TTX-sensitive (TTX-s) or TTX-resistant (TTX-r). The enhanced Na^+ currents in injured axons are largely mediated by TTX-r channels [16]. The NaV1.8 channels, in being both TTX-r and upregulated in injured nerves [17], may be important contributors to this phenomenon. Indeed, ASOs targeting NaV1.8 eliminate the TTX-r component of the C-wave in injured sciatic nerves [18]. Behaviorally, ASOs directed at NaV1.8 have been shown to inhibit both the development and maintenance of nerve injury-induced thermal hyperalgesia and mechanical allodynia [19,20]. In a visceral pain model, NaV1.8 knockdown reduced the hyperexcitability produced by acetic acid treatment in the bladder [21]. Interestingly, as was observed with P2X3, knockdown of NaV1.8 attenuated the behavioral signs of CFA- but not carrageenan-induced inflammatory pain. There may be a fundamental difference in these two models that makes the latter less susceptible to ion channel downregulation. It has been speculated that CFA produces both inflammatory and nerve injury related effects, which, if true, would be consistent with sensitivity of the nerve injury component to the downregulation of P2X3 and NaV1.8.

2.1.3. Other ion channels

Antisense studies targeting other families of ion channels have resulted in the following observations: (1) the NMDA receptor subtypes R1 and R2C are important in the etiology of formalin-induced nociffensive behaviors whereas the R2D is not [22,23]; (2) the 5HT$_7$ receptor is important in 5HT mediated spinal analgesia [24]; (3) the α4 subunit of the nicotinic acetylcholine receptor, nAchR, plays a critical role in nicotinic agonist-induced analgesia [25] and (4) Kv1.1 potassium channels are likely to be involved in morphine, baclofen and clonidine antinociception but not in the antinociception induced by H1 antihistamines [26–28]. This pattern of involvement of Kv1.1 is consistent with the mechanism of action of these compounds (i.e., morphine, baclofen and clonidine are agonists at inhibitory receptors whereas antihistamines are antagonists at an excitatory receptor).

2.2. G-Protein coupled receptors

2.2.1. Opioid receptors

An enormous amount of progress has been made in the opioid receptor (OR) field because of antisense technology. Although there are now knockout mice available, ASO based methodologies pre-date their development and have been applied to a wide range of questions including: identification of functional subtypes, splice variants, and ligand selectivity. There has been an inconsistency in the literature with regard to the OR subtypes in that the pharmacologically-identified subtypes of mu-, delta- and kappa-opioid receptors (δOR, μOR, and κOR) outnumber the cloned receptors MOR1, DOR1 and KOR1. Targeting of the cloned ORs with antisense has supported the existence of pharmacological subtypes at the molecular level. For example, the antinociception elicited by the δOR agonists DPDPE and deltorphin II displays differential sensitivity to ASOs targeting the cloned DOR1 receptor, consistent with previous evidence for the two pharmacological subtypes, δOR-1 and -2 [29–33]. Similarly, the actions of a κOR agonist in the modulation of cutaneous versus visceral nociception were differentially sensitive to KOR antisense treatment, suggesting the presence of an additional, non-KOR1 site of action for this agonist in viscera [37]. ASO-based studies have also supported a role for DOR1 in morphine tolerance, dependence and stress-induced analgesia [34–36]. Finally, ASOs have been used to "map" splice variants by the differential targeting of exons [30]. In this manner it was determined, for example, that different splice variants of the cloned mu-opioid receptor MOR1 display differential sensitivity to a plethora of μOR agonists [38–46].

2.2.2. α_2-Adrenergic receptors

Antisense studies investigating the relative contributions of the α_2-adrenergic receptor (α_2AR) subtypes α_{2A}AR and α_{2C}AR in α_2-adrenergic agonist-induced analgesia are consistent with the knockout studies demonstrating that most [47–49], but not all [50] of the α_2-adrenergic agonists require the α_{2A}AR for analgesic efficacy. In fact, ASO treatment and knockout mice were used in the same study to provide convergent evidence for the involvement of the α_{2C}AR in the analgesic action of the α_2AR agonist moxonidine [50].

2.2.3. Metabotropic glutamate receptors

The therapeutic potential of metabotropic glutamate receptor antagonists and lack of subtype-selective agents have prompted the evaluation of the role of the mGluR1 subtype in pain and analgesia models using ASOs. In acute pain assays, mGluR1 ASO treatment produced analgesia and decreased neuronal activity in response to noxious stimuli but did not alter responses to innocuous stimuli [51]. In chronic pain models, reduction of mGluR1 attenuated nerve injury- and CFA-induced thermal hyperalgesia and mechanical allodynia [52,53]. In addition, some restoration of morphine efficacy was observed in neuropathic rats [52] and mGluR1 ASO treatment attenuated the development of morphine tolerance [54]. Based on these data, mGluR1 antagonists would be expected to reverse hyperalgesia and allodynia and increase nociceptive thresholds and could therefore be effective therapeutic agents. In addition, co-administration of mGluR1 antagonists with morphine may enhance analgesic efficacy and reduce the development of morphine tolerance.

2.2.4. Other G-protein coupled receptors

Several galanin receptor subtypes have been cloned, raising questions regarding their role in different aspects of galaninergic transmission. Antisense studies targeting GalR1 have demonstrated that this subtype is critical for galanin-mediated inhibition of C-fiber-induced facilitation of nociceptive reflexes [55,56]. Thus, GalR1 may mediate the analgesic actions of galanin receptor agonist in the spinal cord. GPCR antisense studies have also shown that the $5HT_{1B}$ serotonergic receptor subtype may not be required for 5HT-mediated analgesia [24] and that cholecystokinin (CCK) has anti-opioid activity at the CCK_B receptor [57]. Other GPCRs which have been successfully targeted with antisense in pain-relevant studies include: muscarinic M1- [58,59], dopamine D2- [60], neurotensin NT1- [61–63] and the cannabinoid CB1-receptors [64,65].

2.3. G-Proteins

Stimulation of G-protein coupled receptors can lead to the activation of multiple G-protein α-subunits (G_α) [66]. Given the functional consequences of differential G_α activation, it is important to understand which of these subtypes is relevant to signaling in vivo. Many of the G_α subunits have been targeted with ASOs and these studies have resulted in some interesting insights into G-protein coupling. Most relevant to pain and analgesia are the studies investigating the complement of G_α subunits activated by specific analgesics. Activity of the supraspinally administered μOR agonists morphine, DAMGO and sufentanil, for example, was attenuated by antisense targeting $G_{i2\alpha}$, but not $G_{i1\alpha}$, $G_{i3\alpha}$ or $G_{s\alpha}$ [67]. In addition, $G_{i2\alpha}$ activation differentiated between morphine analgesia and other effects of morphine such as acute dependence and constipation [68]. It has also been shown that different μOR and δOR agonists are differentially sensitive to knockdown of a given G_α subunit. For example, morphine and morphine-6 β-glucuronide appeared to utilize different G_α subunits [69] as did agonists acting at the δOR-1 and δOR-2 subtypes [70]. Furthermore, analgesics acting at other families of inhibitory GPCRs (i.e., clonidine at α_2ARs) shared G-proteins with some ORs and not with others. For example, Karim and Roerig demonstrated that spinal analgesia elicited by clonidine and morphine was attenuated by knockdown of different G_α subunits [71]. Many of these studies have been reviewed elsewhere [72,73]. In addition to selective knockdown of G_α subunits, the G protein subunit $\gamma 2$ has been targeted with ASOs, revealing a significant role for this protein in antinociception induced by i.c.v. administration of the δOR agonist DPDPE [74].

2.4. Miscellaneous signaling molecules, growth and transcription factors

In addition to the functional roles of many ion channels, GPCRs and G-proteins, ASOs have been applied to a wide range of pain-relevant questions such as morphine tolerance. It has been shown, for example that knockdown of β-arrestin attenuates both morphine tolerance and nerve-injury induced allodynia [75], that knockdown of the nNOS splice variants attenuates the development of morphine tolerance (nNOS-1) or reduces morphine analgesia (nNOS-2) [76] and that PKCα is involved in the development of morphine tolerance. Other molecular classes successfully targeted with antisense include growth and transcription factors. For example, ASO targeting helped identify the role of neurotrophin-3 in nerve-injury induced sprouting [77] and showed that knockdown of c-fos can inhibit inflammation-induced regulation of neuropeptide gene expression [78].

3. Mechanism of action

Antisense inhibition of gene expression relies upon the rules of nucleic acid base pairing. An ASO, typically 15 to 25 nucleotides in length, is designed to bind to a complementary sequence on the target RNA. As a consequence, the protein product coded by that particular RNA is not synthesized. The mechanisms by which ASOs inhibit protein expression fall into two major categories: cleavage of mRNA by activation of RNase H and steric blockade [79,80]. The contribution of these mechanisms to the action of individual ASOs is influenced by their chemistry and the location of the targeted sequence within the RNA molecule.

RNase H is an enzyme that degrades the RNA strand of a DNA–RNA duplex. Upon binding of ASOs to RNA, an RNA–DNA duplex is formed and RNase H digests the RNA that the antisense compound has hybridized with. As the original antisense compound remains intact, the ASO is free to target and bind with another strand of RNA. This process can be repeated over and over, allowing one oligonucleotide (ON) to cause the cleavage of multiple RNAs [79,80]. Two types of mammalian RNase H enzymes have been identified: RNase H1 is thought to participate in replication whereas RNase H2 may play a role in transcription [81]. The limited information available on the relative contribution of the two enzymes to antisense effects suggests that RNase H2 is the major player and its cellular localization is likely to differ in different cell types [82]. The relevance of RNase H-dependent degradation of ASO-targeted genes in the central nervous system (CNS) has been addressed in a study showing that the in vivo activity of ASOs against CRF2 was lost when the ASOs were modified to no longer support RNase H activation [83]. However, the expression, activity and role of RNase H in neurons have not been examined directly.

Non-RNase H-dependent mechanisms of ASO-mediated knockdown are typically ascribed to steric blockade of RNA. Binding of an ASO to RNA has been proposed to interfere with numerous RNA processing events, possibly leading to one or more of the following: inhibition of 5′-capping, modulation of splicing, inhibition of 3′-polyadenylation, translational arrest and the disruption of critical RNA structure(s) [79,80,84,85].

On a practical level, it is important to be aware of the different mechanisms of ASO action. First, some DNA analogues result in the formation of ASO–RNA duplexes which are not substrates for RNase H. Thus, a sequence may show activity when synthesized using one type of DNA analogue but not another. Second, if an ASO elicits its effects solely through steric mechanisms it may reduce expression of a target protein without affecting mRNA levels. Therefore, validation of knockdown limited to mRNA measurement would fail to detect any ASO activity. Finally, ASOs acting through non-RNase H-dependent mechanisms may have effects other than knockdown of expression. Examples include inhibition of the expression of one splice variant while enhancing the expression of another [85] and potentiation of expression of a targeted gene through increased RNA stability [84].

4. Comparison to other gene and protein targeting methods

Alternative technologies for inhibition of protein expression or function include the use of ribozymes, RNAi, viral vectors, sequestering antibodies and

knockout and/or transgenic animals. Ribozymes are somewhat analogous to ASO in that they rely on complementary base pairing for targeting and have the ability to control both the onset and termination of the treatment period. However, unlike ASOs, they also act as enzymes, directly cleaving the target RNA. The use of ribozymes in vivo is largely unexplored. The potential of RNAi for long-term gene silencing has been demonstrated in mammalian systems in vitro [86,87] and in vivo [88] has stirred excitement about its application as a functional genomics tool. However, we are very much at the beginning of exploring the utility of RNAi, particularly in the CNS. Direct comparison of RNAi- and ASO-mediated knockdown suggested many similarities between the two approaches [89]. Use of viral vectors to modulate gene function continues to grow in popularity as the methodologies become more approachable. Viral vectors have been applied successfully in pain-related studies (for a review see Ref. [90]). One disadvantage of this approach is the inability to terminate activity, although this limitation may become less relevant with continued advances in gene delivery methodologies. Sequestering antibodies have been used successfully in pain models (for example see [91]) but their availability is limited.

Currently the most common method for the evaluation of gene function in vivo is the use of knockout and/or transgenic animals. Table 1 outlines some of the characteristics of antisense technology in comparison to knockout and/or transgenic models. This comparison is not meant to favor one method over the other but rather to present some of the relative advantages and disadvantages of each. The ongoing debate regarding the superiority of knockdown vs. knockout and other methodologies should be tempered by the following realizations:

- All methods have inherent difficulties and limitations.
- The decision to pursue one particular experimental approach over others depends on many complex, case-specific factors.
- Alternative methodologies should be viewed as complementary.

5. Experimental design of in vivo antisense-based studies

As is true with any method, success requires a good understanding of the technical advantages and limitations of the technology and problems are nevertheless inevitable. Many groups have invested resources in ASO-based experiments and have been frustrated by uninformative, uninterpretable, misleading or simply irreproducible results. The failure of the ASO technology to live up to its potential can be attributed in large part to the plethora of technical

Table 1
Knockdown versus knockout/transgenic

Antisense technology	Knockout/transgenic technology
Applicable in a wide range of species, including humans	Currently limited to a few species (i.e., mice, pigs)
Effects are fully reversible	Effects are not generally reversible with current technology
Less expensive	More expensive
Rapid: experiments can begin within weeks of conception	Development can be very slow, particularly when accounting for back-crosses
Does not require use of special equipment or facilities	Requires use of a lot of specialized facilities
No confounding developmental effects	Potential exists for confounding development effects
Some risk for confounding compensatory effects	High risk for confounding compensatory effects
Potential for simultaneous targeting of multiple genes	Targeting of multiple genes possible but laborious
Usually only achieves partial knockdown	Total obliteration of gene product
Animals must be generated de novo for each experiment but once a protocol is established it can be easily adapted to other targets	Once a line is established the number of subjects is restricted only by breeding and genotyping expenses but is limited to one target
Need to control for non-specific DNA effects	Need to control for developmental and compensatory effects
High doses of antisense can have toxic effects	Knockout of some genes produces a lethal phenotype
Cannot generate over-expressers	Generation of over-expressers is common
Can be used to target a gene before, during or after an experimental manipulation	Although under development, conditional knockout technology is not yet mainstream

issues that must be addressed when designing an ASO-based experiment. Among the critical issues are sequence activity and selectivity, ON stability, proper use of controls, route of administration and uptake, dose-dependence, time–course and adequate evaluation of knockdown. Careful consideration of all these technical aspects is essential for successful ASO-based studies. Table 2 presents examples of experimental design parameters, the resultant knockdown in expression and the in vivo models used for selected pain-relevant studies from the discussion in Section 2. It is intended to illustrate the many variations in target class, design, application and efficacy of knockdown. The abbreviations used in Table 2 are defined in the relevant topical segments throughout Section 5.

5.1. Antisense oligonucleotide sequence selection

Of the many factors influencing the success of antisense treatment, identification of active ASO sequence(s) is the most critical and cannot be overemphasized. Improved methodologies can only potentiate the activity of weaker sequences that would otherwise fall below detection levels [92]. Estimates suggest that between 5 and 15% of randomly selected ASOs will be sufficiently active at a target gene to generate meaningful results [93–96]. This is largely due to the inaccessibility of most of the RNA molecule for ASO binding as a consequence of its complex secondary structure. Furthermore, associations with cellular proteins will render additional regions unavailable for binding. Although there are methods that increase the probability of finding active sequences, the current state of knowledge is such that at some level sequence identification remains a hit or miss process.

A comprehensive review of the literature by Tu et al., showed that 82% of published antisense studies (up to September 1997) reported data from just a single ASO [96]. In addition, often there is no information on the process or rationale for the selection of the sequence, suggesting that the ASO design was based on a "lucky strike" rather than a systematic approach. The seeming incongruity between this statistic and the probability of randomly hitting upon an active sequence has raised concerns about the integrity of many of those studies [93], yet this judgment may be overly harsh for several reasons: First, there is an inevitable publication bias as studies using inactive ASOs are unlikely to be published because no insights into biological processes will have been obtained. (This situation should not be confused with biologically active ASO studies in which knockdown is achieved but no phenotype is observed). Second, it is generally not common practice to report the inactive ASOs that were rejected before an active sequence was found, just as a medicinal chemist may not report all the derivatives of a compound that lack activity.

The most conservative strategy for ASO sequence identification is the process of gene walking. An in vitro assay is first established to quantify mRNA and/or protein expression of the target. A series of ASOs complementary to the target RNA are then designed by walking along the gene. The entire sequence is covered and all the ASOs are screened for biological activity. Those that show activity may then be further evaluated for specificity, potency and maximal efficacy. It is not uncommon in industrial settings for hundreds of ONs to be screened per gene. In a recent report by Sewell et al., 264 sequences were screened to identify an optimal anti-TNFα ASO [97]. One of the major hurdles encountered in this strategy is the development of an efficient screening system. This requires a suitable cell line that either endogenously expresses or can be transfected with the target gene, the optimization of ON transfection conditions in that cell line and an assay for the detection of knockdown. In addition, the expense of synthesizing the many ON sequences required by this method may be cost prohibitive.

A novel strategy developed by Hoen et al. may facilitate the in vitro evaluation of antisense sequences [95]. The authors created a green fluorescent protein (GFP) fusion construct of their target gene. This construct was then transfected into a cell line. Exposure of the cells to ASOs simultaneously reduced the expression levels of the target gene and GFP. Thus GFP serves as a reporter for ASO-mediated decreases in the expression of the target gene. Major advantages of this method are that it can be applied to any gene independent of its function and that the evaluation of knockdown (i.e., flow cytometry for GFP) is rapid, quantitative and does not require development of a novel gene-dependent

assay. However, the possibility remains that the addition of GFP may change the secondary structure of RNA, resulting in different accessible sites than the native gene.

It is important to note that in vitro efficacy does not necessarily predict nor guarantee in vivo efficacy. The RNA structure, the complement of accessory proteins and the regulation of the target gene may be different in vivo than in the in vitro screening system. This is especially true if the selection is performed using a cell free system where none of the normal RNA-binding proteins are present. Furthermore, the percent activity demonstrated in vitro might not predict the percent knockdown in vivo. Specific details such as the cell line, the type of ONs (i.e., unmodified vs. chemically modified DNA), the efficiency of uptake and the dose will all impact the level of activity in vitro. Similarly, the interaction of complex factors such as chemical modifications, dose, route of administration and diffusion will profoundly influence the degree of antisense activity in vivo. If, for example, a series of potential ASOs are tested and the best one is only able to reduce expression by a third, it is possible that the same sequence might be significantly more efficacious in vivo or under different in vitro conditions. Thus, the use of arbitrary cutoff (i.e., 50 or 75%) to discard ASOs with low in vitro activity levels may lead to the exclusion of active ASOs The potential for discrepancies between in vivo and in vitro efficacies does not discount the possible benefits of selecting active ASOs in an in vitro system provided such a system is easily available, is optimized and models closely the cell types to be targeted in vivo (for example, using neuroblastoma cell lines for CNS targets). Under these conditions failure to detect in vivo activity of ASOs that were active in vitro would be likely due to other factors such as dose or uptake and efforts can therefore be focused on addressing these other variables.

Central to the identification of active ASOs is the characterization of accessible sites on the RNA of the target gene [92]. Computer-based strategies have been developed that involve assessment of the free energy requirements for hybridization and evaluation of computer-predicted local RNA secondary structure [98–100]. In addition, a number of empirical methods have been described, typically based on the use of random ON libraries or arrays to identify accessible regions on the target RNA [101–105]. There are also approaches for identification of active ASO that are independent of characterization of accessible sites. Several groups have performed comprehensive literature surveys to identify sequence motifs which are positively or negatively correlated with antisense activity [96,106]. In one study the sequence motif GGGA was found in a disproportionate number of successful sequences When this motif was then used to select ASOs to a specific gene, the proportion of active ASOs increased from 2/18 when randomly selected to 18/25 [96]. Some of these computer-based tools have been made available on-line (for example, see Refs. [107,108]).

Over the years the experimental use of ASOs has resulted in the generation of "rules" for sequence design including: avoid sequences with G-quartets, avoid sequences that contain the two-base sequence CpG (cytosine–phosphate–guanine), and favor ASOs which will hybridize in proximity to the start codon. G-Quartets (i.e., four G-bases in a row) result in the formation of a quadruplex which interacts with a number of proteins, causing non-specific effects [79]. Systemically administered phophorothioate ASOs containing the sequence CpG activate mammalian B cells and natural killer cells [109]. Immune system stimulation has therefore limited their use as antisense drugs. Interestingly, this immune response is only triggered when the CpG sequence is unmethylated. It is hypothesized that because the unmethylated version is common in bacterial DNA and not in mammalian DNA, the immune system interprets the unmethylated ON sequences as bacterial and activates a response to combat the "bacterial" invasion. In an interesting twist, these properties may be exploited for possible therapeutic benefits [110,111]. It should be noted, however, that the relevance of these phenomena to centrally administered ONs is not clear. Finally, the belief that ASOs directed near the area of the start codon are more likely to be active has yet to be substantiated by systematic studies. The seemingly disproportionate representation of such ASOs in the literature could easily be accounted for by the ubiquity of that particular strategy rather than any advantages that it may confer.

When ultimately deciding how to approach ASO design, it is worthwhile to investigate the services of commercial vendors. Considering the potential losses in productivity (it may divert a significant proportion of time from other projects), the ensuing distraction from the real biological questions and the expense of developing a reliable screening assay, the commercial price tags may seem more reasonable. In some cases, commercial vendors also offer access to proprietary DNA chemical modifications which may be advantageous.

5.2. Antisense selectivity

Next to the identification of a biologically active ASO, sequence selectivity is the second most important factor to be considered in the design and interpretation of antisense studies. Part of the allure of antisense comes from claims of absolute selectivity, yet there is surprisingly little evidence to support this contention. On the contrary, evidence exists which presents considerable challenges to this assumption. Woolf et al. studied the effect of mismatches (substitution of bases that are not complementary to the target RNA) on the activity of ASOs in *Xenopus* oocytes [112]. Whereas an intact 25-mer produced 79% degradation of the target mRNA, sequences with 17/25 and 14/25 matches resulted in 32 and 37% degradation, respectively. An unrelated control produced 13% degradation. Thus, mismatch of nearly half the sequence failed to completely eliminate ASO activity. The same study also concluded that as few as 10 consecutive complementary bases are sufficient to produce cleavage of a target RNA and that this is not prevented by mismatched flanking sequences. Thus, any 10-base stretch within an ASO could hybridize to any complementary 10-base stretch of RNA and produce non-selective effects. The implications of this study regarding the selectivity of antisense treatment are alarming. Hypothetically, assume that a 20-mer generally requires 6/20 base switches for complete loss of efficacy (a number commonly found in the literature). One must, therefore, also assume that a given 20-mer could potentially hybridize to and block the expression of any mRNA with which it has 15 or more matches. Add to that the fact that any given 20-mer will contain 11 10-mers, each capable of binding to complementary RNA and non-selective antisense effects on genes that may or may not be related to the targeted gene become a very real possibility. Whether this theoretical prediction presents grounds for concern in practice is difficult to assess at present. A thorough evaluation of potential non-selective antisense targeting would require a comparison of all possible combinations of (up to six) mismatches and 10-mers against the database of available sequences while taking into account RNA accessible sites. A further complication arises from the fact that the genome of the rat, the species of choice for many antisense studies, is not completely characterized. From a practical standpoint, the use of "gapmer" ASOs (see Section 5.3), where the length of the RNase H-sensitive sequence is limited, can potentially reduce the risks of non-selective effects.

Based on current standards, an ASO is generally considered to be selective if (a) it produces a functional effect, (b) mRNA or protein knockdown of the target is demonstrated and (c) control ONs have no effect on function or expression levels. This case is considered to be strengthened by the demonstration that the expression and/or function of a completely unrelated gene is unaffected, but this observation only provides support for a lack of general toxicity or disruption of RNA synthesis following ON administration. In some cases it may not be possible to selectively target a specific subtype within a closely related family if it has limited unique regions and these regions are not sensitive to ASO treatment. In such cases, however, it may be possible to target a subset of the family members (i.e., subunit 1 and 2 but not 3) or to develop a pan-antisense which targets the entire family (for example, see Ref. [31]).

In addition to inadvertent non-selective targeting due to sequence homology, knockdown of a target may be associated with changes in the expression, trafficking or function of other related or unrelated genes due to mechanisms such as co-regulation. This may result in assigning to the target functional properties that should be attributed to "non-selectively" affected genes. It is therefore important that the demonstration of knockdown of the target is accompanied at a minimum by evaluation of closely related genes. At present, the vast majority of

published papers fail to provide any evidence that closely related family members are unaltered by ASO treatment. This is particularly problematic in cases where the family members are known to have similar function and the goal of the study is to isolate the function of a single member from its close relatives [50,113–115]. The number of members in the family and/or the lack of appropriate tools may impose practical limitations on the number of relatives that can be examined. However, the identity of the family members most likely to complicate interpretation can be narrowed down significantly by taking into account what is known about their anatomical distribution and function. The Na^+ channels NaV1.8 and NaV1.9, for example, are the only TTX-r subunits expressed in sensory neurons [116] and upregulation of TTX-r currents in injured nerves contributes to their hyperexcitability [16]. Therefore, the demonstration that ASOs targeting NaV1.8 but not NaV1.9 had functional consequences in neuropathic pain models [20] suggests that the injury-related changes in TTX-r channels can be attributed to NaV1.8. In addition, the expression of two TTX-sensitive relatives was not altered by NaV1.8 ASO treatment [20]. Although a stronger case could have been made if NaV1.9 protein levels were also shown to be unaffected by ASOs targeting NaV1.8, the within family controls described above greatly substantiate the interpretation of the findings.

The level of complexity increases when considering the consequences of targeting a member of a protein family that forms functional heteromers. The loss of a heteromeric "partner" could have profound impact on the remaining partners, causing changes in expression, trafficking and subunit composition [117–119]. In the P2X family of ion channels, subunits P2X2 and P2X3 are known to form heteromers in sensory neurons. P2X3 homomers are also found in these cells but P2X2 homomers are not [2]. The role of the P2X3 subunit in chronic pain has been evaluated in both antisense and knockout studies. Changes in the expression level of the P2X2 subunit were not evaluated in any of these studies [10–13]. Without controlling for changes in P2X2 protein expression, many of the findings presented in these reports could have alternative explanations. For example, if P2X2 requires P2X3 for proper trafficking to axon terminals where it produces a functional effect, then it may be incorrect to attribute all functional changes directly to P2X3. Furthermore, loss of a subunit may cause formation of aberrant homomeric or heteromeric channels. It has been shown for both the $GABA_A$ and the NMDA receptors that ASO-mediated knockdown of certain subunits modulates the composition and properties of the remaining channels [117,118,120,121]. The resultant functional differences could therefore be due to the appearance of inappropriate channels rather than to the loss of a single subunit.

The adequate evaluation of antisense selectivity has been hampered in part by the daunting magnitude of performing expression analysis of a large number of proteins. Recently developed approaches for large-scale expression profiling at the RNA and protein level offer the opportunity for comprehensive and efficient evaluation of the global effects of antisense treatment [122,123]. DNA microarray studies have reported ASO-mediated changes in the expression of tens to hundreds of genes in addition to the targeted transcript [124,125]. These changes were attributed to hybridization of the ASO to homologous sites [124] as well as secondary effects on genes that may be functionally modulated by the targeted gene [125]. Understanding the global effects of antisense treatment is critical for its application as a functional genomics tool in drug discovery because they may impact the ability to predict the therapeutic potential of novel drug targets. Furthermore, the ability to analyze the global effects of ASO-mediated knockdown of pain-relevant proteins offers a novel application of ASOs to the study of pain and analgesia. For example, knockdown of a growth factor or a component of a second messenger pathway is likely to interfere with the expression of proteins regulated by the targeted gene. Therefore, large-scale analysis of protein expression following ASO treatment could provide information on signaling pathways downstream from the target gene.

5.3. *Oligonucleotide chemistry*

The use of unmodified phophodiester (PDE) ONs is generally not recommended. Their high susceptibility to nuclease degradation, resulting in a very short half-life, has limited their utility in most applications. The CNS, however, represents a special

Table 2
Examples of experimental design and outcome measures

Family (target)	Experimental design (chemistry, delivery, dose, controls)	In vivo assay(s)	Evaluation of knockdown	Ref(s)
Ion channels				
P2X (P2X3)	2′-MOE gapmer with PDE or PS backbone Slow infusion (i.t.) 24–240 μg/day (rat) Universal or four-base MM	Spinal nerve ligation and sciatic nerve injury Peripheral inflammation Formalin α,β-ATP-hyperalgesia	~50% ↓ mRNA in DRG ~20% ↓ in staining by IHC in spinal cord ~35–45% ↓ in DRG by Western blot (range = 0–65%)	[12,13]
NaV (1.8, 1.9)	Phosphodiester (PDE) 2× daily injection (i.t.) 90 μg/day (rat) Six-base MM	Spinal nerve ligation Peripheral inflammation Visceral (bladder) pain Na^+ currents in DRG and sciatic nerve	~45% ↓ in DRG by Western blot ~50–75% ↓ by IHC in DRG by cell count or intensity ↓ by IHC in nerve (qualitative)	[18–21]
NMDA (R1, R2C, R2D)	Phosphodiester (PDE) 2× daily injection (i.t.) 10 nmol/injection (rat) Sense & scrambled	Formalin	20–40% ↓ in NMDAR1, ~30% ↓ in NMDAR2C ~40% ↓ in NMDAR2D of mRNA by RNase protection in spinal cord	[23]
5HT $5HT_3$	Phosphodiester (PDE) One injection per 48 h (i.t.) 20 μg/injection (mouse) Four-base MM	5-HT-agonist induced analgesia	$5HT_3$ binding was undetectable in ASO treated group	[24]
G-Protein coupled receptors* α_2-Adrenergic receptors (α_{2A}AR, α_{2C}AR)	Phosphodiester (PDE) 2× daily injection (i.t.) 15 nmol/injection (rat, cervical) 12.5 μg/injection (mouse) Sense, four base MM	α_2AR agonist-induced inhibition of NMDA or substance-P evoked behaviors	25–50% ↓ by IHC in spinal cord	[47–50]

Table 2 Continued

Family (target)	Experimental design (chemistry, delivery, dose, controls)	In vivo assay(s)	Evaluation of knockdown	Refs.
Metabotropic glutamate receptors (mGluR1)	PS-endcapped PDE Slow infusion (i.t.) 6–50 μg/day (rat) Sense, four-base MM	Acute pain—tail flick & dorsal horn activity CFA-induced inflammation Sciatic nerve injury Morphine tolerance	15–35% ↓ by IHC Large range by Western blot analysis (~30–100%)	[51–54]
Galanin receptors (GalR1)	Peptide nucleic acids 2× daily injection (i.t.) 1.5 nmol/injection (rat) Scrambled	C-fiber-induced facilitation of nociceptive transmission	26% ↓ in Galanin binding by autoradiography	[55,56]
5-HT receptors (5-HT_{1B})	Phosphodiester (PDE) One injection per 48 h (i.t.) 20 μg/injection (mouse) Four-base MM	5-HT-agonist induced analgesia	70% ↓ in binding	[24]
Miscellaneous nNOS	Phosphodiester (PDE) One injection per 48 h (i.c.v.) 5–20 μg/injection (mice) Four-base MM	Morphine tolerance Morphine analgesia	~50–80% ↓ by mRNA ~35% ↓ in enzymatic activity	[25]
β-Arrestin	Phosphodiester (PDE) 2× daily injection (i.t.) 5 μl of 2 nM/injection (rat) Four-base MM	Morphine tolerance Nerve injury-induced allodynia	60% ↓ in mRNA (compared to MM control)	[35]
Neurotrophins	Fully phosphorothioated Slow infusion (i.t.) 0.6 nmol/day (rat) Sense controls	Nerve-injury induced sprouting & allodynia	~45% ↓ in spinal cord by ELISA	[57]

* ORs are not included in the table due to the volume of relevant publications

case in which unmodified PDEs are stable enough to produce antisense effects. This is perhaps due to reduced nuclease activity in the CNS compared to peripheral tissues and serum. However, even in the CNS it is often necessary to deliver PDEs by continuous or repeated dosing to maintain active concentrations. Significant efforts have been made towards the development of modified ONs with increased stability, enhanced RNA binding affinity and reduced toxicity. Some issues to consider when evaluating the suitability of a particular chemical modification include binding affinity, stability, toxicity, uptake, mechanism of action, cost and availability. Of the hundreds of modifications that have been developed, those with the most relevance to the CNS are discussed below.

3′-Inverted

One approach to increase ON stability has been to invert the polarity of the terminal bases [83]. The assumption is that the 3′-inverted end would be less susceptible to exonuclease-mediated ASO degradation. The benefits of this modification have not been conclusively demonstrated.

Phophorothioates

The development of ONs in which the PDE bonds between nucleotide bases are modified to form phosphorothioate bonds has resulted in ONs with increased resistance to enzyme degradation while retaining RNase H induction. Often referred to as PS-ONs, phosphorothioate ONs have been very useful in in vitro systems and have been successfully applied in many in vivo studies.

PS-ONs are more potent than PDE-ONs in the CNS [83,126]. In a comparative study PS-ONs and 3′-inverted PDE-ONs targeting the corticotrophin releasing factor type-2 receptor were administered intracerebroventricularly (i.c.v.). Whereas a single injection of 0.5 nmol resulted in a 50% reduction in CRF binding, PO-ONs needed to be given twice daily at a 10-fold higher dose to produce a 42% decrease. Unfortunately, PS-ONs suffer from a number of serious limitations. They are notorious for having multiple non-antisense effects, probably due to their documented binding to many extracellular, cell surface and intracellular proteins [79]. To further complicate matters, these non-antisense effects may exhibit some sequence dependence, which can therefore easily be mistaken for biological effects one might expect from inhibiting the target mRNA. The most serious problem, however, is their toxicity in CNS. For example, i.c.v. administration of PS-ONs results in fever, weight loss and various symptoms of ill health [83,127,128]. These effects are independent of nucleotide sequence but are dose-dependent. In addition, intraparenchymal delivery of high doses of PS-ONs results in severe tissue damage characterized by cell loss and the appearance of lymphocyte-like cells in the damaged region [126]. PS-ON use in the CNS is therefore contraindicated.

Endcap PDE-PS chimeras

In an attempt to maintain some of the beneficial qualities of PS-ONs while reducing toxicity, chimeric ONs in which the first and last few bases are linked by PS bonds while the rest of the backbone is PDE have been tested. The rationale for "endcapping" is that the terminal PS linkages confer resistance to exonuclease activity. Endcap ONs exhibit similar efficacy to fully phosphorothioated ONs with reduced toxicity, although they do result in some cell damage [126]. In a related approach, the middle eight bases of a PDE-ON were replaced with PS linkages to protect the ASO against endonuclease activity. These chimeras were well tolerated for up to 9 days and demonstrated significant efficacy [83]. In another study, both endcap ONs and a 15-mer with five PS residues in the center showed signs of toxicity following intraparenchymal injection, albeit less severe than fully phophorothioated ONs [129]. Thus, although PDE-PS chimeras have higher efficacy than PDEs and are less toxic than PS-ONs, the toxic effects limit their utility.

Peptide nucleic acids (PNAs)

PNAs are ON analogues in which the deoxyribose phosphate backbone is replaced by a neutral polyamide backbone. They are resistant to nuclease activity and form more stable DNA/DNA and DNA/RNA complexes [130]. Initially it was thought that the utility of PNAs would be limited by poor cellular uptake. Efforts were therefore made to couple them to transporter peptides to enhance uptake. This approach was used successfully to decrease expression of the galanin receptor GalR1 following in-

trathecal (i.t.) delivery of PNAs [55]. It was later shown, however, that PNAs could be delivered intracellularly to neurons without transporter peptides following direct injection into the PAG [62] and that i.t. administered unmodified PNAs could inhibit GalR1 without uptake enhancers [56]. Thus, uptake into neurons did not appear to be a limitation of PNAs. Furthermore, it was demonstrated that systemically (10 mg/kg; i.p.) administered PNAs could cross the blood brain barrier and reduce gene expression in the PAG [63]. However, in another study using a transgenic mouse model in which ASOs correct aberrant splicing of EGFP (enhanced green fluorescence protein), systemically administered PNA-ASOs did not have a CNS effect [131]. Finally, PNAs are not substrates for RNase H and their effects may be more complex than just knockdown of the targeted gene. For example, PNA ASOs have been shown to alter the splicing of the interleukin-5 receptor-alpha chain; the expression of the membrane-bound isoform was inhibited whereas that of the soluble one was enhanced [85].

Locked nucleic acids (LNAs)

LNAs are ribonucleotides in which the 2′-oxygen of ribose is connected to the 4′-carbon by a methylene bridge. LNAs dramatically increase the stability and binding affinity of ASOs [132]. The melting temperature of an unmodified PDE ASO targeting DOR1 increased from 59 °C to >90 °C when fully LNA-modified [129]. Although fully LNA-modified ASOs do not activate RNase H, LNA:DNA:LNA gapmers (see below) are potent activators of RNase H. LNAs have been delivered to the CNS by i.t., i.c.v. and intrastriatal administration [129]. Spinally delivered LNA:DNA mixmers and gapmers were found to be more potent than unmodified PDE-ONs and fully-LNA modified ONs showed no signs of toxicity following intraparenchymal administration [129].

Gapmers

Gapmers are ONs which consist of a PDE or PS center flanked by modified nucleotides. The modified ends of the gapmers result in increased stability and/or RNA affinity while the stretch of PDE or PS monomers in the center confers ability to activate RNase H. Chemical modifications that have been used for gapmer design include LNA, PNA, and replacement of the hydrogen at the 2′-position of ribose by a methyl (2′-OMe) or methoxyethyl (2′-MOE) group [132,133]. Whereas a center stretch of seven or eight PS monomers are required for LNA:DNA activation of RNase H, 2′-OMe gapmers require only six [132]. To date there is no evidence of gapmer-mediated toxicity. LNA and 2′-MOE gapmers have been used in vivo in pain-related studies [12,13,129]. Although the 2′-MOE modification is not available for general use, the related 2′-OMe modification is commercially available and 2′-OMe gapmers have been shown to target successfully the capsaicin receptor VR1 in vitro [132].

5.4. Controls

Controls are a crucial component of any experimental design. In antisense studies, inactive DNA sequences are used to control for the specificity of the treatment. There is currently little to no agreement regarding the most appropriate DNA controls. It is well known that ON administration may have non-specific effects in addition to the desired antisense-mediated knockdown in expression of the target gene. Many of these effects are difficult to predict and/or explain given our current knowledge and are highly dependent on the specifics of each experiment (e.g., chemistry, route of administration, dose).

Administration of ONs to the CNS, regardless of sequence, may result in increased body temperature, reduced fluid intake, weight loss, decreased motor activity, changes in nociceptive thresholds, neuronal loss, induction of interleukin-6 and infiltration of lymphocyte-like cells as well as other signs of general sickliness [83,126,129,134,135]. The nature and intensity of these effects varies with many factors such as dose, route and duration of administration and ON chemistry. The inclusion of the proper controls is therefore essential for the interpretation of antisense experiments.

Oligonucleotide effects can be sequence-dependent, sequence-related and sequence-independent [136]. Sequence-dependent effects include hybridization to the target mRNA and the inadvertent targeting of homologous mRNAs. Sequence-related effects do not have an antisense-based mechanism but are

associated with specific DNA motifs and/or the formation of secondary or higher-order structures, such as guanosine-quartets, CpG motifs or palindromic sequences [93,109,137]. Sequence-independent effects arise from the chemical properties of the ONs. The use of the term non-specific effects often encompasses all effects of an ON not attributable to an antisense-based action at the target gene. However, since sequence-dependent and sequence-related effects may have a specific mechanism of action, such as affinity for a related mRNA, the term non-specific will only be used here to describe sequence-independent effects. It should be noted that the contribution of sequence-independent effects to the observed effects of ASO-treatment can only be adequately evaluated based on comparison of the DNA control group to a vehicle control group. The importance of vehicle controls is further discussed in Section 5.7.

Many types of DNA controls have been used in

Table 3
Advantages and disadvantages of DNA controls

	Advantages	Disadvantages
Mismatch	Can perform mismatch vs. function studies May control for problematic motifs Control for physical properties such as size and base composition	Potential residual hybridization at target Potential hybridization at non-target genes Possible appearance of problematic motifs Results may be difficult to interpret Difficult to design
Scrambled	Will not hybridize with target mRNA Control for physical properties such as size and base composition	Potential hybridization at non-target genes Appearance of and/or failure to control for problematic motifs Difficult to design
Reverse	Will not hybridize with target mRNA May control for some problematic motifs (if not orientation sensitive) Control for physical properties such as size and base composition Easy to design	Potential hybridization at other genes Appearance of and/or failure to control for problematic motifs
Sense	Easy to design	Potential hybridization at other genes High incidence of unexplained biological activity Appearance of and/or failure to control for problematic motifs
Universal	No design necessary Excellent control for sequence-independent effects	Not well represented in literature Failure to control for problematic motifs and most other physical properties such as base composition Potential for confounding effects unexplored
Targeting of unexpressed or unrelated genes	Control for sequence-independent effects	Failure to control for problematic motifs and most other physical properties such as base composition An "unrelated" or "unexpressed" gene may turn out to be "related" and/or "expressed"
Non-expressing cells	Useful in vitro to demonstrate selectivity of ASO	Not useful in vivo
Additional ASO sequences	Provides multiple converging lines of support for experimental findings	Increased use of resources Difficult to find multiple unique active sequences against a target gene
Isosequential modified ASOs as controls	Control for non-specific effects	The use of additional chemistries may require changes in protocols and could become very labor intensive

antisense studies and each one has its own peculiar share of advantages and disadvantages. It is therefore important when designing or evaluating antisense studies to be cognizant of the limitations of the different types of DNA controls. In general, DNA controls should be of the same length and chemistry as the ASO and should lack homology to other known genes. In addition, motifs such as G-quartets must be equally present or absent in both antisense and control ONs. Table 3 and the discussion below highlight some of the key issues to consider for the commonly used DNA controls.

Mismatch oligonucleotides as controls

Mismatch (MM) DNA controls, in which several pairs of bases within the antisense sequence have been swapped, are common in the literature. The primary advantage of MM controls is the retention of many properties of the active ONs such as molecular weight and GC content. In addition, MM ONs may retain problematic sequence motifs and higher-order structures characteristic of the ASO sequence. Thus, both sequence-related and sequence-independent effects may be accounted for.

The main disadvantage of MM controls is the potential for residual activity at the target gene, which can compromise interpretation of the results. If, for example, an experiment is performed in which a MM control produces a partial effect, it would not be possible to distinguish between the following possibilities: (1) the partial effects are due to residual affinity at the gene of interest (sequence-dependent). (2) The partial effects are due to the inadvertent targeting of a related or unrelated gene (sequence-dependent). (3) The partial effects are due to a specific motif that may or may not be present in the ASO sequence (sequence-related). (4) The partial effects are due to general toxicity of the compound itself (sequence-independent).

The potential for residual hybridization at the target gene raises the question of how many bases need to be switched to eliminate target-specific hybridization. As discussed above in Section 5.2, it has been suggested that mismatch of nearly half the sequence may fail to eliminate the ASO activity [112]. In another study a mRNA was targeted by four different active ASOs, each with their own mismatch controls. While all four ASOs inhibited mRNA levels, two of the MM controls had partial efficacy and two lacked efficacy, highlighting the variable nature of MM controls. The number of switches necessary to eliminate hybridization is currently theoretically unpredictable and will depend on many complex factors, including the affinity of individual ASO for their RNA binding site, the location of the mismatches (mismatches at the 5′ or 3′ ends are less effective than those in the middle), the type of base (some substitutions will have greater effects on hybridization affinity than others) and the total number of bases swapped.

In light of the limitations described above, the use of MM ONs as controls is therefore not advisable. In the event that it is necessary to use MM ONs to account for a problematic motif, examination of a series of MM ONs with increasing numbers of switches should ideally be employed. A correlation between the number of misplaced bases and loss of function strongly supports a sequence-specific mechanism of action for the ASO [138,139]. Alternative approaches include generating multiple MM controls, in which independent base pairs are swapped or combining MM with another type of control (i.e., Universal, see below). An unavoidable consequence of the above-mentioned strategies, however, is an increase in the expense of each experiment.

Scrambled oligonucleotides as controls

A variation of the mismatch approach is to design a control ON by completely scrambling the ASO sequence. Scrambled ON controls, in maintaining the GC content of the ASO, share many of the advantages of MM controls but are free of lingering questions regarding residual hybridization at the target or closely related genes. However, they do share some of the other disadvantages of MM controls such as the unpredicted inadvertent hybridization to unrelated genes. A limitation of scrambled controls is the failure to control for problematic motifs that may be present in the ASO such as G-quartets.

Reverse oligonucleotides as controls

Reverse ONs are prepared by synthesizing an ASO sequence in reverse. The reverse control for an ASO reading 5′-ATCCG-3′ would be 5′-GCCTA-3′. Reverse controls share the same advantages and

disadvantages with scrambled controls but are easier to design. However, since the sequence is predefined by the ASO sequence, the use of reverse controls is limited by their potential homology to other genes.

Sense oligonucleotides as controls

Sense controls are those complementary to the ASO and identical to the mRNA target. The sense control for an ASO reading 5′-ATCCG-3′ would therefore be 5′-CGGAT-3′. There are many disadvantages to their use including the failure to control for problematic motifs (i.e., GGGG becomes CCCC). There is a prevailing view that sense controls produce biological effects at a higher frequency than can be attributed to sequence-independent effects alone [136]. Despite the lack of hard data supporting this notion, sense controls have little advantage over other control sequences and therefore can be avoided

Universal or "randomer" oligonucleotides as controls

Universal control DNA consists of a mixture in which the probability of an A, T, G or C being inserted at each position is equal. The result is a mixture of low concentrations of all possible sequences. This mixture is an excellent control for non-specific effects such as DNA toxicity. Other advantages include ease of design and the ability to control for multiple antisense sequences simultaneously. However, our lack of knowledge regarding the potential effects of low levels of DNA hybridizing to many mRNAs simultaneously is a cause for concern.

Antisense targeting unexpressed or unrelated genes as controls

The inclusion of an ASO known to be active against a gene not expressed in the experimental system can be used as a control (i.e., targeting luciferase in rat spinal cord). A negative result with an ASO that is active in other systems is considered by some to provide stronger support for selectivity than would a scrambled or reverse control. However, from the perspective of the spinal cord, a sequence with activity at luciferase looks no different from scrambled except in its failure to control for GC content-related factors such as molecular weight or hybridization strength. These controls are therefore less advantageous than scrambled or reverse. In an extension of this strategy, genes that are expressed in the experimental system but are thought not to be involved in the phenomenon under investigation could be used as controls. In the event that knockdown of the unrelated gene is validated yet has no functional effect in the experimental assay, specificity will have been well demonstrated. However, identification of a candidate "inert" gene is problematic and in the event that the control has partial efficacy the experiment would be uninterpretable.

Non-expressing cells or knockouts as controls

One can test for non-specific effects of an ASO in a system where the target gene is not present. This is particularly applicable to in vitro systems in which the target gene is being artificially expressed. In such cases, the effect of ASO-treatment in untransfected cells can be compared to those expressing the target gene. Any additional effects observed in the transfected cells would be considered to be antisense-mediated and sequence specific. However, this experiment would not control for the potential emergence of undesired sequence-dependent effects in vivo. Consider, for example, a situation in which genes closely related to the target mRNA are expressed in vivo but not in the in vitro system. The potential for cross-reactivity between related genes will be missed in vitro. In addition, in vitro assays will not control for non-sequence dependent effects that might arise in vivo such as toxicity at the site of administration. There are currently no published reports of ASO-treatment in a knockout animal. Ideally, the antisense should have no activity compared to DNA controls since the target gene is absent. Any other result would be difficult to interpret.

Additional antisense sequences as controls

The ability to demonstrate similar effects with multiple ASOs targeting the same gene strongly supports an antisense-based mechanism of action *if and only if* the potential for non-specific effects has been convincingly eliminated with appropriate DNA controls.

Isosequential antisense oligonucleotides as controls

If the same ASO, synthesized with two different

chemistries, decreases expression of the target gene and produces a consistent biological phenotype in independent experiments, it would be considered highly unlikely that the ASO-mediated effects were non-specific. However, this type of experiment should be considered complementary to and therefore only used in addition to proper DNA controls.

5.5. Route of administration

ONs are unable to penetrate the blood–brain barrier and are delivered to the CNS either i.t., i.c.v. or intraparenchymally using chronic indwelling catheters. Due to limited diffusion [140–142], these approaches target a relatively small area in proximity to the catheter or cerebral ventricles. Another major disadvantage of these approaches is their invasiveness, which can result in non-specific effects. Behavioral assays are particularly sensitive to the stress and discomfort associated with invasive surgeries. Potential non-invasive alternatives for antisense administration to the CNS include intranasal delivery [143–145] and systemic delivery of stable chemically-modified ASO to the CNS [61,63,146,147]. Finally, transcutaneous administration may be useful for delivery to sensory neurons via their peripheral terminals [148].

Intrathecal delivery is the most common route of administration used in pain-related antisense studies. It is usually accomplished via a chronic indwelling catheter [149], although many groups employ direct lumbar puncture, particularly in mice [150]. The primary limitations of the direct lumbar puncture method for antisense studies are the need for repeated injections and the skill of the injector. Since the injector should be blind to the treatment groups and no clear functional readout for successful injection is immediately available, the injector must have demonstrated a consistently high ($>90\%$) success rate.

In rats and larger animals i.t. delivery is almost always accomplished using catheters. The exposed end of the catheter can be used for bolus injections or can be attached to an osmotic pump for slow infusion over time. A direct comparison between bolus injections and minipump delivery of ASOs noted significantly greater ASO uptake in dorsal root ganglia (DRG) cell bodies following slow infusion [13]. This method also requires less involvement of technical staff and causes less stress in the animals. There are two main approaches to catheter placement. The most commonly used is to extend the catheter from the cisterna magna to the rostral end of the lumbar enlargement. Although animals catheterized in this manner do not have overt pain symptoms, there are signs of compression injury to the spinal cord as well as formation of scar tissue around the end of the catheter [151]. A more recently developed method inserts a catheter at the level of the lumbar enlargement using direct lumbar puncture [152]. This method is preferable for both humane and scientific reasons but is currently more labor intensive.

The position of the catheter relative to the dorsal and ventral horns of the spinal cord varies considerably. Given the observation that dorsally placed catheters result in ASO effects in dorsal but not ventral horn [51], ventrally placed catheters are unlikely to result in knockdown of proteins expressed in the superficial dorsal horn. Therefore, inclusion of animals with ventrally or laterally placed catheters is likely to contribute to increased variability in pain-related antisense studies. The location of the catheter can be determined using lidocaine prior to functional analysis [54] or using post-mortem analysis [51] and only animals with dorsally placed catheters should be included in the analysis.

5.6. Oligonucleotide uptake

ASOs are readily taken up in the CNS without the aid of uptake enhancers. The presence of ASO in sensory neurons following i.t. administration has been visualized using fluorescent probes and antibodies recognizing chemical modifications [13,19]. However, the series of events that deliver the antisense from its point of infusion to the sensory neurons as well as the factors that influence these events are unclear. Although the extent of diffusion of ASOs within the i.t. space is uncharacterized, it appears that the ASO effect is restricted to the region caudal to the catheter [19]. The effect of the rate of infusion is unknown, but this factor is likely to influence the effective concentration of ASO at the site of action throughout the treatment. The precise site(s) of neuronal uptake of ASO after i.t. administration has not been determined; possibilities include the central terminals in dorsal horn, the dorsal roots

or the DRG cell bodies. Furthermore, the relative degree of ASO uptake in DRG neurons versus spinal cord neurons has not been characterized. However, uptake of fluorescently tagged ONs has been shown in spinal cord [22] and antisense has been used to successfully target proteins expressed in dorsal horn [23,50], indicating that ASOs have access to spinal cord neurons. From a practical standpoint, factors that should be considered when choosing a probe for the visualization of ASO delivery include the stability of the ON and the stability of the probe under the experimental conditions (e.g., structural integrity at body temperature, resistance to enzymatic cleavage, compatibility with fixation).

Comparison of the in vivo uptake efficiency of ASOs with different chemical modifications may help elucidate these mechanisms and optimize the experimental design of antisense studies. For example, in vitro it has been demonstrated that in the absence of uptake enhancement, cationic (e.g., PNAs with four lysines at the 3′ end) and neutral (morpholinos) ASOs cross the cell membrane more efficiently than negatively charged ASOs (2′-MOE) [153]. Increased uptake of PNAs containing 4 lysines at the 3′ end has also been demonstrated in vivo [131].

Systematic comparisons of technologies designed to enhance cellular uptake of DNA in vivo are needed to optimize ASO delivery. Liposomal carriers are commonly used in vitro to facilitate uptake and many of these preparations are commercially available. Due to differences in their properties, it may be necessary to test several sources before identifying the best preparation for each in vitro system [89]. Although it has been shown in at least one study that liposomes did not facilitate ON uptake in the CNS [22], comprehensive screening of different preparations would be required to identify a potentially useful one. Furthermore, given our incomplete understanding of the mechanisms of ON uptake in the CNS, it is not possible to predict if the use of liposomes will confer any advantage.

An interesting strategy for uptake enhancement is the conjugation of ONs to carriers that may facilitate endocytosis. The conjugation of ONs to cell penetrating peptides has been shown to enhance ON delivery to the nucleus in vitro [154]. The potential of these methods for in vivo delivery in the CNS, however, remains to be fully explored. Furthermore, there is evidence that these peptides contribute to non-specific effects [124]. In another approach, biotinylated ONs were bound to a transferrin receptor monoclonal antibody-streptavidin (mAb-SA) conjugate [146,147]. Upon binding to the antibody, the transferrin receptor underwent receptor mediated endocytosis across the blood brain barrier, delivering systemically administered ONs into the CNS [146,147]. An alternative strategy, which may contribute to increased uptake as well as cell-specific targeting, is to conjugate the ON to a ligand which binds to a receptor expressed by the targeted cell type, thus inducing endocytosis. In one example, ONs were covalently bound to a glycoprotein which caused receptor-mediated internalization in the target cells [155]. It should be noted that the benefits of increased uptake of carrier-ON conjugates may be compromised by intracellular trafficking such as trapping in endosomes, which would limit the access of ONs to their site of action [156].

5.7. Oligonucleotide dose

In the famous Indian fable "The Blind Man and the Elephant", a group of blind men were assembled and each was presented a part of an elephant: the head, ears, tusk, trunk, foot, back, tail or tuft, and then asked to describe what sort of thing an elephant is. In describing just the part of the animal that they had access to, their descriptions were all simultaneously partially correct and completely wrong. In pharmacology, conclusions based on analysis of a single dose point are analogous to the blind men's descriptions of the elephant: simultaneously partially correct and completely wrong. In the pain-relevant literature there are surprisingly few studies examining the effects of ON dose and dosing schedule in vivo. As a result, the impact of these factors on antisense treatment in the CNS is unknown. It is known, however, that the therapeutic window might be narrow and that the dose range and ideal dosing schedules will change with chemistry, route of administration, the target gene and the experimental endpoint.

The importance of examining the full dose range of ON effects is illustrated in the hypothetical experiment presented in Fig. 2 and Table 4. In this example, the effect of an ASO is compared to one of

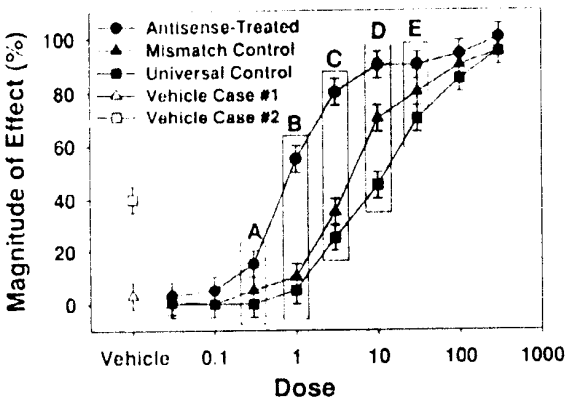

Fig. 2. Hypothetical data demonstrating the importance of dose–response analyses. The vertical grey bars delineate the % response at a single dose point. Depending on the dose examined, the results may lead to different conclusions, as outlined in Table 4. The results may also be interpreted differently depending on the response of a vehicle control group or in the absence of such a control.

two DNA control groups—a MM and a "universal" control. As shown in Table 3, this experiment would lead to fundamentally different conclusions depending on the presence of a vehicle control, the baseline set by a vehicle control and, most critically, the dose point examined. In the absence of a vehicle control group, evaluation of dose B or C would suggest the presence of an ASO-mediated effect whereas doses A and E would indicate a lack of effect in all ON-treated groups. In the presence of a vehicle control, examination of a single dose-point leads to an entire range of conclusions (no effect, ASO-specific effect, non-specific DNA effects) depending on the chosen dose.

Our current lack of appreciation for the importance of ASO dose is illustrated by examining the effects of i.t. ASOs targeting P2X3 in three independent studies. In one study, antisense and control ONs were synthesized as 2′-OMe gapmers with a PDE middle [157]. The 2′-OMe chemistry is related to the 2′-MOE chemistry used in the other two studies [12,13]. In all three studies ONs were administered by slow infusion through osmotic minipumps. The doses used and percent knockdown were as follows. (1) Doses of 2.4, 7.2 and 24 μg/day of 2′-OMe-PDE gapmers all resulted in an approximately 30% decrease in P2X3-immunoreactivity (P2X3-ir) by quantitative image analysis in the dorsal horn [157]. (2) Approximately 30% knockdown in P2X3-ir in dorsal horn and 50% in P2X3 mRNA measured by in situ hybridization in DRG was achieved using 2′-MOE-PDE gapmers at 180 μg/day [12]. (3) 2′-MOE-PS gapmers at 240 μg/

Table 4
Experimental interpretations vary with dose and vehicle controls

	Dose	Antisense	Mismatch	Universal	Conclusion
Relative to DNA controls	A	–	–	–	Inactive ASO
	B	+	–	–	ASO-specific effect
	C	+	–	–	ASO-specific effect
	D	+	+ / –	–	ASO effect, non-specific DNA effect*
	E	–	–	–	Inactive ASO
Relative to vehicle control 1 (5% effect)	A	–	–	–	Inactive ASO
	B	+	–	–	ASO-specific effect
	C	+	+ / –	+ / –	ASO effect, non-specific DNA effect
	D	+	+ / –	+ / –	ASO effect, non-specific DNA effect
	E	+	+	+	Non-specific DNA effects
Relative to vehicle control 2 (40% effect)	A	(+)	(+)	(+)	Non-specific DNA effects*
	B	+	(+)	(+)	ASO effect, non-specific DNA effect*
	C	+	(+)	(+)	ASO effect, non-specific DNA effect*
	D	+	+ / –		ASO effect, non-specific DNA effect*
	E	+	+	+	Non-specific DNA effects*

* These non-specific DNA effects are only observed in the MM control group.
* These non-specific DNA effects are bi-directional.

day produced an average of 40% knockdown by Western blot analysis [13]. The magnitude of P2X3 knockdown is therefore relatively constant from 2.4 and 240 μg/day. The apparent "saturation" of the knockdown could be rationalized in multiple ways, such as the relative efficacy of the sequences used and the possibility that intrinsic regulatory mechanisms limit the antisense effect to a maximum of 30–50%. Regardless, these data emphasize the need for systematic analysis of antisense effects in terms of dose dependency.

A final note about dose: the doses of ON delivered into the CNS are best expressed in terms of nmol and not μg. As the molecular weight of ONs changes with base composition, length and chemical modification, two samples, each with the same number of μg per unit volume, might have significantly different ON concentrations. Therefore, it would be technically incorrect to assume that groups of animals treated with equal volumes of these samples have been exposed to the same dose. When converted to nmol per unit volume, the concentrations are standardized across samples and direct comparisons can be made.

5.8. Dosing schedule

The level of protein expression and the rate of turnover will impact the efficacy, time course and dose requirement of antisense treatment. The longer the half-life of the protein, longer time–courses of administration may be required. For example, functionally relevant knockdown of G-protein α-subunits and β-arrestin can be achieved with single ASO injections [67,75]. In most protocols targeting GPCRs, animals are dosed for up to 3 days yet the half-lives are thought to range from 2 to 8 days [83]. Longer treatment periods may result in significant increases in knockdown. When ASO treatment against CRF_2R was extended from 5 to 9 days, the reduction in receptor binding increased from 40 to 60% to 80% [83].

In addition to the total length of treatment, the treatment interval chosen could have dramatic consequences on the results of a study. Fig. 3 depicts the effect of three different dosing schedules (12, 24 and 48 h intervals) on a hypothetical endpoint. The arrows indicate each time compound is administered. In this example, if the experimental endpoint is measured 12 h after the first treatment, similar effects would be observed in all three groups. If the first assay point is 24 h after the beginning of treatment, an effect will be observed in the 12 h group but not the others. If the measurements are taken 36 h following initiation then efficacy will be observed in the 12 and 24 h interval groups but not the 48. The purpose of this illustration is to stress the importance of the time-course of action following administration. In practice it may therefore be advantageous to give small doses several times daily or slow infusion rather than expose the animals to larger doses less frequently. This should reduce acute toxicity and maintain ASO concentrations at a more steady level. Furthermore, ASOs delivered i.t. by slow infusion have been shown to accumulate to much greater extent in DRG cell bodies than following repeated bolus injection [13].

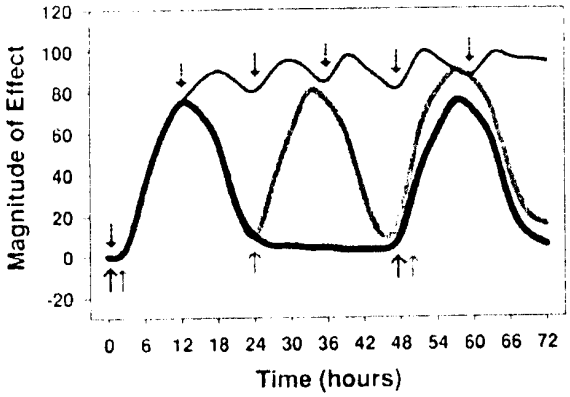

Fig. 3. Illustration of the effect that different dosing schedules may have on antisense efficacy. Each arrow represents an administration. The drug is administered every 48 (thick black; ↑), 24 (dark grey; ↑) or 12 h (thin black; ↓). Larger treatment intervals may introduce substantial fluctuations in the magnitude of the ASO-mediated effects. As a result, the effect of ASO-treatment may be missed if the experimental endpoint is not measured at the optimal time.

5.9. Evaluation of knockdown

The demonstration of antisense-mediated knockdown of the targeted gene is necessary for the interpretation of antisense studies. In the presence of knockdown, lack of a functional effect after antisense treatment would be interpreted as evidence that the targeted gene has no functional role in the

studied phenomenon. However, in the absence of knockdown, the lack of functional effect would be attributed to insufficient biological activity of the ASO and would provide no information about the function of the target gene. Similarly, without demonstration of knockdown, any functional changes observed after ASO-treatment cannot be attributed to ASO-mediated effect at the target gene.

In many antisense studies in the literature the magnitude of the functional changes exceeds that of the change in expression. For example, a 35–55% reduction in deltorphin II binding was associated with 80–90% reduction in deltorphin II-mediated analgesia [129]. Similarly, reduction of P2X3 expression by less than 50% resulted in the complete loss of αβmeATP-induced mechanical hyperalgesia and greater than a 50% decrease in αβmeATP-induced flinching behaviors [12,13]. This discrepancy is most likely associated with phenomena such as spare receptors and with the fact that the functional assay and the expression analysis may measure different pools of the targeted gene.

In addition to its importance for interpretation of functional results, systematic analysis of knockdown performed prior to functional analysis can be very beneficial in the design of the functional portion of the study. Information on the dose-dependence and time–course of changes in expression of the targeted gene provides a basis for selecting an appropriate dose range and dosing schedule for functional studies. It could be argued that performing expression analysis prior to functional analysis is not an efficient use of time and resources. However, expression analysis requires less animals per experimental group than functional studies. In the long run this approach may therefore save time and resources that would have been wasted using inactive ASOs or performing functional analysis at suboptimal dose and/or time-points.

The issues surrounding controls, dose and time–course discussed in the preceding sections are as relevant to the analysis of knockdown as they are to the functional analysis. In a number of studies the control MM ASO resulted in increased variability in the expression levels of the targeted gene compared to other experimental treatments (for example, see Refs. [19,54]), consistent with the possibility of residual efficacy of the MM. However, in the absence of other DNA controls it is impossible to determine if MM effects are due to residual efficacy at the target gene or to non-specific effects. Moreover, in some of these studies statistical significance is reported based on comparison of the antisense group to the vehicle group using a *t*-test, without comparison between the antisense and the mismatch group. In order to conclusively demonstrate an antisense-mediated effect, it is necessary to demonstrate that the antisense group is different from both the vehicle and the control DNA group(s) using statistical tests for multiple comparisons (for example, see Ref. [23]). Finally, there have been surprisingly few studies exploring the dose-dependence and time–course of antisense-mediated knockdown (for examples, see Refs. [71,158]). A correlation between the degree of knockdown and the functional effects across dose and/or time would support a causative link between them.

Antisense-mediated knockdown can be evaluated at the mRNA or the protein level, and ultimately the approach chosen is determined largely by the available tools and expertise. Levels of mRNA can be assayed by a variety of methods, including Northern blot analysis, in situ hybridization, RNA protection assay or real-time reverse transcription polymerase chain reaction (RT-PCR). Many antisense studies assess knockdown solely based on mRNA measurement (for example, see Ref. [23]). However, antisense treatment reduces mRNA levels only if the ASO acts through an RNase H-dependent mechanism, and therefore limiting the expression analysis to mRNA levels may result in a false negative. In fact, ASOs that act through non-RNase H-dependent mechanisms may even enhance the levels of the targeted gene [84,159]. Furthermore, the relationship between mRNA level and protein expression is often non-linear [122]. Given these difficulties, relying on mRNA assays to validate knockdown could lead to misinterpretation of the experimental results.

Ideally a comprehensive antisense study would include expression analysis at both the mRNA and the protein level (for example, see Refs. [12,126,160]). If this is not feasible, it is preferable to demonstrate decreases in protein levels because they will ultimately determine the levels of functional activity. The majority of methods for analysis of protein expression are antibody-based, including

immunohistochemistry (IHC) (for example, see Refs. [19,161]), Western blot analysis (for example, see Refs. [13,71,160]), enzyme-linked immunosorbent assay (ELISA) (for example, see Ref. [77]) and radioimmunoassay (RIA) (for example, see Ref. [126]). In all of these approaches, the quality of the results will first and foremost be limited by the quality of the antibody used. Non-antibody based methods such as receptor binding (for example, see Refs. [24,162,163]), autoradiography (for example, see Refs. [22,56]), or mass spectrometry are also available.

Evaluation of knockdown requires some form of quantitative analysis. Qualitative judgments based on visual observations are generally not accepted as proof of antisense-mediated effect. Based on the type of tissue processing, the methods for expression analysis fall into two major categories: histological (e.g., in situ hybridization, IHC, receptor autoradiography) and biochemical (e.g., Northern blot, Western blot, ELISA, RNA protection assay, real-time RT-PCR, mass spectrometry). Histological approaches offer the advantage of focusing the analysis on the relevant area. However, the quantification of histological observations relies on image analysis, which is not very sensitive, can be subjective and may be confounded by high variability due to tissue processing. Quantitative image analysis inevitably requires selection of a labeling threshold, which distinguishes specific signal from background. Although it is sometimes possible to establish a protocol for objective selection of threshold [164], it is more common for the observer to set the threshold subjectively based on the labeling in control groups. Strategies for minimizing the effects of variability and subjectivity include: (1) process samples from different experimental groups in parallel so that each group is represented on a given slide; (2) determine the threshold based on the labeling in the vehicle control group; (3) average measurements from several slides to obtain the final values for each subject; (4) perform the analysis blind to the experimental conditions.

Unlike histology, the tissue collection protocols associated with biochemical methods do not usually allow for precise microdissection of the region most likely to contain changes in expression. This could result in the dilution of any antisense effect, making it harder to observe changes. However, with the exception of methods based on electrophoresis and densitometry (Northern blot and Western blot), this disadvantage is outweighed by the superior sensitivity and, in some cases, the ability to measure absolute amounts in individual samples. Using an RNA protection assay, Yukhananov et al., determined that treatment with ASO targeting the NMDA2RD subunits decreased expression of the subunit from ~1.5 to ~0.75 amol/μg of total RNA (1 amol = 10^{-18} mol) [23]. A relatively new approach for RNA quantification is real-time RT-PCR, which measures mRNA levels based on the number of replication cycles necessary to detect the gene of interest in the sample relative to a housekeeping gene [165]. In its most commonly applied form, real-time RT-PCR measures are relative amounts, but methods for determining absolute amounts based on standard curves are under development [166]. This approach has been used for quantification of antisense-mediated knockdown [160,167] although due to its novelty, its representation is the literature is limited.

At the protein level, quantitative evaluation of knockdown by ELISA is particularly feasible in the case of neurotrophins [77], for which there are commercially available tools that have been applied in numerous experimental paradigms, including transgenic animals [168]. However, the measurement of absolute expression levels using RNA protection or ELISA requires the generation of a standard curve of known amounts of mRNA/protein, which is usually not trivial. An additional limitation in the use of ELISA is the requirement for high-quality antibodies. A more predominant method for quantification of knockdown at the protein level is receptor binding (for example, see Refs. [24,162,163]). It is sensitive as well as technically straightforward. However, just as ELISA is dependent on antibodies, receptor binding (and autoradiography) is limited by the quality and availability of ligands. Since the motivation for many antisense studies is the lack of selective ligands for functional distinction of subtypes, suitably selective ligands may not be available. The use of non-selective ligands for receptor binding is an option [56] although the presence of additional non-ASO-targeted binding sites will dilute the results, making it difficult to detect knockdown.

Advances in proteomic analytical methods based

on mass spectrometry offer new approaches for knockdown evaluation [169–173]. The advantages of mass spectrometry for protein quantification are its superior sensitivity in the fmol range and the ability to assess protein levels directly, independent of a ligand or antibody. Until recently, the application of mass spectrometry to analysis of complex protein mixtures from biological systems has been restricted by the limited capacity of two-dimensional gel electrophoresis (the predominant separation strategy) for detection of low abundance and transmembrane proteins [174]. This limitation has been overcome by the introduction of multidimensional liquid chromatography as an alternative separation strategy [171–173]. In addition, the use of isotope-coded affinity tags (ICATs) has allowed measurement of relative differences in protein levels between two samples by mass spectrometry [122,169,170]. Although the application of these methods to the mammalian nervous system is just beginning to be explored and is likely to be challenging, their adaptation to antisense studies may facilitate the analysis of ASO-mediated knockdown of protein expression.

6. Conclusions

The use of ASOs has contributed greatly to the field of pain and analgesia. Nevertheless, ASO-based approaches have failed to meet the high expectations initially placed upon them. This failure can be largely attributed to an underestimation of the complexity of antisense technology and a lack of appropriate tools for its successful application and evaluation. Over the past decade, sustained exploration has lead to an increasing understanding of the pitfalls encountered in in vivo ASO-based studies. This accumulated knowledge, in combination with continued technological developments, have set the stage for a long-overdue, systematic and in-depth analysis of ASO-mediated effects in vivo. Empirical and in silico methodologies for the selection of active sequences and the development of new chemistries have improved the design, specificity and stability of ASOs. Sensitive methods for the evaluation of ASO-mediated knockdown have enabled quantitative assessment of the dose- and time-dependency of action. As a result, our ability to design and interpret the results of functional studies has improved dramatically. Technologies for large-scale expression analysis at the RNA and protein level allow for global evaluation of both specific and non-specific ASO effects. Taken together, these advances have the potential to increase our understanding of the mechanisms of ASO-based approaches, facilitate the application of ASO as a functional genomics tool, and enable sophisticated approaches for the development of new pain therapies. The promise of antisense-based technology is therefore stronger than ever.

Acknowledgements

We would like to thank Dr. R. Elde and Ms. Jill-Desiree Brederson for their critical reviews of this manuscript and are grateful for our experience at Neuromics, Inc., where we were exposed to the challenges of in vivo application of antisense oligonucleotides.

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